# Clinical and genetic determinants of vitamin D receptor expression in cutaneous melanoma patients

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**Decrease of vitamin D receptor (VDR) expression is observed in melanocytic naevi and melanoma compared to normal skin. Little is known about factors influencing VDR expression in cutaneous melanoma (CM). We investigated the correlation of VDR expression in CM with 25-hydroxy vitamin D (25OHD) levels, demographic/clinical parameters, genetic variants of *VDR* and pathology of the primary tumor. Demographic/clinical parameters were recorded in 407 prospectively recruited CM patients of a multi-center controlled study (ViDMe trial). We determined VDR expression both in the nucleus and in the cytoplasm by semi-quantitative assessment in CM tissue using histochemistry in 279 patients, expressed in percentages and histoscore (H-score). Genomic DNA from 332 patients was extracted to genotype thirteen *VDR* single nucleotide polymorphisms (SNPs) using TaqMan. VDR expression in CM tissue from 279 patients was correlated with clinical/demographic parameters and 25OHD levels (univariable and multivariable analysis), *VDR* SNPs (univariable analysis) and pathology parameters of primary CM tissue (univariable analysis). Cytoplasmic VDR expression was increased in patients who stated to have a high sun exposure during their life compared to patients with low sun exposure ($p_{H\text{-score,univariable}}$: 0.001, $p_{H\text{-score,multivariable}}$: 0.004). The A allele of the genetic *VDR* polymorphism Fok1 was associated with a higher expression of the VDR in the cytoplasm ($p_{cytoplasmic,\ univariable}$: 0.001 and $p_{H\text{-score},\ univariable}$: 0.02). In the primary tumor, presence of mitosis ($p_{nucleus,\%,\ univariable}$: 0.002) and perineural invasion ($p_{nucleus,\%,univariable}$: 0.03) were significantly associated with low nuclear VDR expression. ClinicalTrials.gov Identifier: NCT01748448.**

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## Introduction

Cutaneous melanoma (CM) is the number one cause of death from skin cancer, with rising incidence rates worldwide during past decades. CM caused in 2020, an estimated 3.6 mortality rate (European age-standardized per 100 000) for both sexes in Belgium [1]. Melanoma pathogenesis implicates a stepwise transformation of melanocytes to melanoma, with subsequent superficial and deep invasion and metastasis. The strongest risk factors are a family history of CM, multiple benign or atypical naevi, and a previous CM. Additional risk factors are use of immunosuppression, fair phototype and exposure to ultraviolet light (UV). Melanoma is classified into melanoma in situ, confined to the epidermis, or invasive melanoma, with invasion into the dermis. UV exposure through sun exposure and tanning beds is a well-known risk factor for CM. On the other hand, UV radiation is the main source of vitamin D (VD) synthesis. The latter exists in two forms, vitamin D2 (VD2) and vitamin D3 (VD3). VD2 is the plant-derived form and is exclusively obtained through the diet. VD3 can be produced in the skin from 7-dehydrocholesterol after exposure

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to a sufficient amount of UVB or obtained from dietary sources. In the classical metabolic pathway, VD is consecutively metabolized by the liver and kidneys to the active form 1alpha,25-dihydroxyvitamin D (1,25OHD). Other pathways in the human body for the production of 1,25OHD are described [2]. VD acts through activation of the vitamin D receptor (VDR). Measurement of the serum concentration of 25-hydroxyvitamin D (25OHD) is the gold standard for assessing the VD status in the body [3]. The role of VD is controversial: contrary to the negative effects of UV radiation on melanoma pathogenesis, evidence suggest that VD plays a beneficial role in several malignancies, including CM. Laboratory data, animal studies, epidemiological observations and clinical studies indicate that VD may affect tumor progression or CM outcome. Several epidemiological studies suggested a better prognosis for CM patients with normal serum VD levels at diagnosis compared to CM patients with low serum VD levels [4]. Higher VD levels are associated with lower Breslow thickness and a better overall survival and progression-free survival [5]. VD exerts its physiological function via the VDR, a nuclear transcription factor belonging to the nuclear receptor superfamily that binds 1,25OHD with high affinity and specificity. Upon binding to 1,25OHD and coreceptor protein retinoid X receptor, VDR translocate from cytoplasm into the nucleus and binds to vitamin D responsive elements (VDREs), which up- or down-regulate hundreds of genes directly controlled by VD. The human *VDR* gene is located on chromosome 12q13.11 and comprises at least 5 promotor regions, 11 exons and 11 introns [6].

VDR is abundantly expressed in the skin and VDR expression has been identified in cultured melanoma cells, in melanoma xenografts and in primary CM tissue. Genetic variants of *VDR* have been associated with outcome of CM and may modulate its actions and expression, with Fok1 (RS2228570), Bsm1 (RS1544410), Apa1 (RS7975232), and Taq1 (RS731236) being the most studied single-nucleotide polymorphisms (SNPs) of the *VDR* [7]. Previous studies have documented a decrease in VDR expression from normal skin to melanocytic nevi and melanomas, suggesting a relationship between VDR expression and melanoma pathogenesis [8,9]. While the prognostic properties of VDR expression in CM are well studied, little is known about factors influencing its expression in CM. Given the central role of VDR in mediating VD activity and the beneficial role of VD on melanoma outcome, the influence of baseline 25OHD levels, clinical/demographic characteristics and common genetic variants of the *VDR* gene on VDR protein expression in the primary tumor was investigated in a prospective recruited melanoma population. In addition, in the same population, the VDR expression in the primary tumor was associated with tumor stage and tumor histology to test VDR as a prognostic marker.

## Methods

### Patient recruitment

Patients were recruited at 4 different sites: the University Hospitals in Leuven, the University Hospital in Antwerp, the Centre Hospitalier Universitaire in Liège and the Clinical Center of the University in Debrecen. In total, 407 patients diagnosed with a CM were included in this study, which is part of a multicenter randomized controlled phase III trial (ViDMe trial). This study was approved by the local Ethics Committees and the complete protocol has been published previously [10]. Main inclusion criteria were patients diagnosed with a CM, stage IA to III according to the 8$^{th}$ edition of the American Joint Committee on Cancer (AJCC) classification diagnosed not longer than 12 months before inclusion, age between 18 and 80 years without any adjuvant treatment before inclusion. Inclusion was allowed after surgery and completion of wound healing. Surgical excision, staging (including sentinel procedure) were done in accordance with international guidelines. The following demographic/clinical parameters were recorded by questionnaire in 407 prospectively recruited CM patients: age, gender, smoking status, educational level and total sun exposure during lifetime. The latter was scored based on a questionnaire where we asked the patients to score their total lifetime sun exposure, sun exposure from outdoor recreation and sun exposure from their job according to the options of very low (=1), low (=2), moderate (=3), high (=4), very high (=5). Total sun exposure used in the analysis was the sum of these 3 scores. A score below 9 was counted as low sun exposure, a score of 9 to 11 was counted as normal sun exposure and a score of 12 or higher was counted as high sun exposure. Current (concomitant) drug intake and calcium supplementation were registered. Patients were questioned about taking VD supplements. Timepoint of start taking VD supplementation and amount was registered. A maximum of 800 international units per month was allowed for the study. A full skin examination was performed by a physician to assess the skin phototype, naevus phenotype, presence of freckles and signs of chronic sun damage. The latter was determined by the presence of actinic keratosis, solar lentigines and guttate hypomelanosis. Patient's height and weight were measured in order to estimate the BMI. A blood draw was performed to determine 25OHD levels at randomization.

### Determination of VDR expression

After inclusion, formalin-fixed and paraffin-embedded tissue from primary melanomas was requested from the laboratory where the primary diagnosis was made. Tissue from 279 CM patients was obtained for VDR expression analysis. In total, 4 sections of µ6 m thick were made and processed for hematoxylin and eosin staining for diagnostic confirmation including serial section for VDR immunohistochemical staining.

The expression of VDR was assessed immunohistochemically in the primary tumor. Sections of 3 µm in thickness melanoma samples were placed in a BOND-MAX Automated Immunohistochemistry Vision Biosystem (Leica Microsystems GmbH, Wetzlar, Germany) according to the following protocol. First, tissues were deparaffinized and pretreated with the Epitope Retrieval Solution 2 (EDTA-buffer pH8.8) at 98 °C for 20 min. After washing steps, peroxidase blocking was carried out for 10 min using the Bond Polymer Refine Detection Kit DC9800 (Leica Microsystems GmbH). Tissues were again washed and then incubated with the primary antibody VDR (rabbit-antibody SAB4503071. SIGMA, Darmstadt, Germany); diluted 1:200, for 30 min. Subsequently, peroxidase activity was developed using 3,3'-amino-9-ethylcarbazole, revealing a bright red color that contrasted well with the brown melanin.

Melanoma specimens were reviewed by pathologists involved in the study but unaware of all other clinical and molecular data during evaluation.

Immunohistochemical assessment was performed in both the nuclear and cytoplasmic compartments of tumor cells. The score in the cytoplasmic compartment was made through Histoscore, which is a numerical value used to summarize biomarker expression in a tissue which considers both the percentage of stained cells as well as the intensity of staining. It is defined as follows:

Histoscore = [1 × (% cells 1+) + 2 × (% cells 2+) + 3 × (% cells 3+)]. The score, ranges from 0 to 300.

Regarding the nuclear compartment, scoring was performed according to the percentage of nuclear staining present in the tumor area.

### Determination of VDR gene polymorphisms

A blood sample (K2EDTA tubes) for DNA analysis was taken at randomization. Genomic DNA was extracted from 332 patients coming from the ViDMe trial. Most patients were white individuals of European ancestry (97%). There is a variation in total number of samples analyzed per *VDR* SNP (Tables 4 and 5).

Thirteen genetic variants affecting *VDR* expression were genotyped using Quantstudio 12k flex by TaqMan allelic discrimination assay: rs9729, rs739837, rs731236, rs7975232 (Apa1; T > G), rs757343, rs1544410 (Bsm1; G > A), rs7962898, rs4760733, rs7967152, rs7975128, rs2228570 (Fok1; C > T), rs10735810 and rs7139166. Fok1, Apa1 and Bsm1 are the most studied polymorphisms of *VDR*. Only samples for which ≥80% of SNPs were successfully genotyped were considered for further analysis. SNPs with a call rate <9% were genotyped again to reach success rates close to 100%. At least 5% of the samples were genotyped in duplicate, to prove accuracy of the genotyping protocol. All SNPs passing the quality control were tested for their association with VDR expression in both cell cytoplasm (expressed in percentages and H-score) and nucleus (expressed in percentages).

### Statistical analysis

Univariable associations between pre-defined clinical, socio-demographic and histopathological parameters and VDR expression in the nucleus and cytoplasm were assessed using the Spearman correlation statistic for continuous and ordinal variables. For categorical variables, the Kruskal–Wallis (KW) test was used. The following biochemical, demographic and clinical parameters of a CM cohort were assessed: baseline 25OHD levels, age, gender, smoking status, BMI, season, Fitzpatrick phototype, VD supplementation, score for intensity of lifetime sun exposure, education level, hair and skin color, eye color, total number of benign naevi, AK, solar lentigines, freckles and guttate hypomelanosis (Table S1, Supplemental digital content 1, *http://links.lww.com/MR/A358*). To further explore the relevance of the significant correlations between VDR expression in the cytoplasm (expressed as H-score) and clinical, socio-demographic parameters and VD supplementation at baseline in univariable analyses (Table 1), a multivariable linear regression analysis was performed (Table 2), including all characteristics that were statistically significant ($P < 0.05$) in the above univariable analyses. To meet the model assumptions (normally distributed residuals and constant variance), variable square-root transformation was applied to the H-score.

Univariable analyses as described above were performed for the following pathological parameters: histological subtype of the primary tumor, Clark level, Breslow thickness, ulceration, mitosis, regression, microsatellites, vascular invasion, perineural invasion and TNM staging (Table 3).

For the genetic association analysis, we used PLINK, a freely available open-source software that offers a comprehensive and well-documented set of automated genetic association quality control and analysis tools [11]. For each SNP, we applied a linear regression model, with VDR expression as outcome (dependent variable), and genotype coded as 0/1/2 (additive model) as independent variable. All tests were two-sided and assessed at a significance level of 5%. Due to the exploratory nature of the study, no adjustment for multiple testing was applied. Analyses were performed using SAS/STAT software, version 9.4 for Windows [12].

## Results

### VDR immunohistochemical staining

All 279 CM tissue had a positive cytoplasmic VDR staining with a median value of 80% (Quartile (Q) 1–Q3: 30–100). The mean cytoplasmic VDR expression was 65.34% (SD: 35.8). Tumors showed a positive nuclear VDR staining on a much smaller scale. The mean nuclear

**Table 1 Correlations between VDR expression and demographic, biochemical and clinical characteristics**

| | VDR % nucleus | | VDR % cytoplasm | | VDR H-score | |
|---|---|---|---|---|---|---|
| Gender | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| Male | 119 | 30 (17–70) | 119 | 85 (40–100) | 119 | 100 (40–140) |
| Female | 159 | 40 (15–70) | 160 | 70 (30–100) | 159 | 90 (30–120) |
| Kruskal–Wallis Test | | $P = 0.81$ | | $P = 0.20$ | | $P = 0.04$ |
| Season | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| Winter | 93 | 40 (20–70) | 94 | 60 (25–90) | 93 | 65 (30–120) |
| Spring | 60 | 40 (20–75) | 60 | 90 (40–100) | 60 | 100 (40–140) |
| Summer | 58 | 40 (20–80) | 58 | 90 (30–100) | 58 | 100 (40–140) |
| Autumn | 67 | 20 (10–70) | 67 | 90 (20–100) | 67 | 100 (30–120) |
| Kruskal–Wallis Test | | $P = 0.03$ | | $P = 0.03$ | | $P = 0.25$ |
| VD supplementation | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| No | 240 | 40 (19–70) | 241 | 80 (30–100) | 240 | 100 (40–130) |
| Yes | 38 | 30 (10–60) | 38 | 43 (20–100) | 38 | 50 (20–110) |
| Kruskal–Wallis Test | | $P = 0.27$ | | $P = 0.06$ | | $P = 0.02$ |
| Total sun exposure | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| Low sun exposure | 163 | 40 (20–70) | 164 | 70 (25–100) | 163 | 80 (30–120) |
| Normal sun exposure | 91 | 30 (10–70) | 91 | 85 (30–100) | 91 | 100 (30–160) |
| High sun exposure | 23 | 50 (30–85) | 23 | 100 (70–100) | 23 | 130 (100–190) |
| Spearman rho (95% CI) | | −0.05 (−0.16 to 0.07) $P = 0.45$ | | 0.17 (0.05 to 0.28) $P = 0.005$ | | 0.19 (0.07 to 0.30) $P = 0.002$ |
| Hair and skin color | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| Light skin, red or red-blonde hair | 23 | 40 (10–70) | 23 | 80 (30–90) | 23 | 90 (30–110) |
| Light skin, blonde or light brown hair | 135 | 30 (10–70) | 136 | 60 (25–100) | 135 | 80 (30–120) |
| Light skin, brown or black hair | 81 | 40 (25–85) | 81 | 90 (50–100) | 81 | 110 (60–170) |
| Medium tone skin, brown or black hair/brown skin, dark brown or black hair/black skin, dark brown or black hair | 38 | 40 (10–80) | 38 | 65 (20–100) | 38 | 78 (25–120) |
| Spearman rho (95% CI) | | 0.13 (0.02 to 0.25) $P = 0.03$ | | 0.07 (−0.05 to 0.19) $P = 0.25$ | | 0.10 (−0.01 to 0.22) $P = 0.08$ |
| Actinic Keratosis | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| No presence | 243 | 40 (10–70) | 244 | 80 (30–100) | 243 | 90 (30–120) |
| Presence | 35 | 50 (20–70) | 35 | 100 (50–100) | 35 | 115 (60–191) |
| Kruskal–Wallis Test | | $P = 0.18$ | | $P = 0.03$ | | $P = 0.02$ |
| Idiopatic guttate hypomelanosis | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| No presence | 249 | 40 (15–70) | 249 | 85 (30–100) | 249 | 100 (40–130) |
| Presence | 29 | 40 (20–60) | 30 | 40 (25–85) | 29 | 60 (30–100) |
| Kruskal–Wallis Test | | $P = 0.99$ | | $P = 0.01$ | | $P = 0.03$ |

VDR expression was 43.67% (SD: 31.01) and median of 40% (Q1–Q3: 15–70). One patient had only a positive cytoplasmic VDR staining and no nuclear VDR staining (Fig. 1).

### Patients and clinical characteristics

In total, 407 CM patients coming from the ViDMe trial were included into the study. From that patient population, VDR expression of 279 patients was determined on CM tissue. The clinical, socio-demographic, pathological and genetic parameters of those 279 CM patients were included in the statistical analysis. Most patients were recruited in Belgium. Average age upon inclusion was 53.9 (SD: 13.3) years and average BMI at baseline was 26.4 kg/m$^2$ (SD: 4.6). A minority of CM patients indicated that they were smoking upon inclusion (15.8%). Most patients were included in the study during autumn and winter. In accordance with the geographical location of recruitment, the majority of the patients had a Fitzpatrick skin type II with light skin, blond or light brown hair color and blue, green or gray eye color. Fourteen percent of the patients were taking a VD supplement at the moment of inclusion, of which 79% were taking supplements at time of diagnosis. Most patients indicated having a low total sun exposure (59.0%). In our study population, 46.6% indicated having a vocation university degree or higher as highest educational level. Upon physical examination, most patients had less than 25 benign naevi. Also, most patients had signs of chronic sun exposure with solar lentigines in head-neck region (75.3%) and shoulders (81.0%). A minority of patients had actinic keratosis (AK) (12.4%), presence of freckles (16.9%), and presence of idiopathic guttate hypomelanosis (10.78%). A

detailed summary of patient demographics is provided in supplementary table S1, Supplemental digital content 1, *http://links.lww.com/MR/A358*.

### Patients and melanoma characteristics

In this study population, most patients had a superficial spreading melanoma (54.8%). Median (IQR) Breslow thickness was 1.37 (1.00–2.00) mm. In most cases, primary tumor tissue had a Clark level IV or higher (63.5%) and presence of mitosis (92.3%). Minority of primary tumor tissue showed presence of ulceration (19.9%), regression (12.6%), perineural invasion (5.8%), vascular invasion (4.64%) and microsatellites (2.7%). Following pathological classification of the patients according to the 8$^{th}$ edition of AJCC for Tumor (T) staging, most patients were at randomization a stage T2a (37.1%), followed by T1b (14.4%) and T3a (11.5%). Of all study patients included, 82.5% had no nodal involvement upon inclusion. For total Tumor-Node-Metastasis (TNM) staging, most patients included in the study presented a stage IB (56.1%), followed by stage III (16.9%) and stage IIA (13.7%).

### Correlation of patients' biochemical, demographic and clinical characteristics with the VDR expression in CM

In univariable analyses, statistically significant associations were found between VDR expression and gender ($p_{H\text{-}score}$: 0.04), season at time of inclusion ($p_{cytoplasmic,\%}$: 0.03; $p_{nuclear}$: 0.03), VD supplementation ($p_{H\text{-}score}$: 0.02), total sun exposure ($p_{cytoplasmic}$: 0.005; $p_{H\text{-}score}$: 0.002), hair and skin color ($p_{nuclear}$: 0.03), AK ($p_{cytoplasmic,\%}$: 0.03, $p_{H\text{-}score}$: 0.02) and idiopathic guttate hypomelanosis ($p_{cytoplasmic,\%}$: 0.01, $p_{H\text{-}score}$: 0.03). These data indicate that melanomas of patients who stated to have a high sun exposure during their life and patients with AK display enhanced cytoplasmic VDR expression, whereas a reduced VDR cytoplasmic expression was found in female patients, patients taking VD supplements and patients with idiopathic guttate hypomelanosis. Concerning nuclear VDR expression, a decrease was significantly associated to patients with blond/light

**Table 2 Correlations between VDR expression in the cytoplasm expressed by H-score and demographic, biochemical and clinical characteristics, multi-variable analysis**

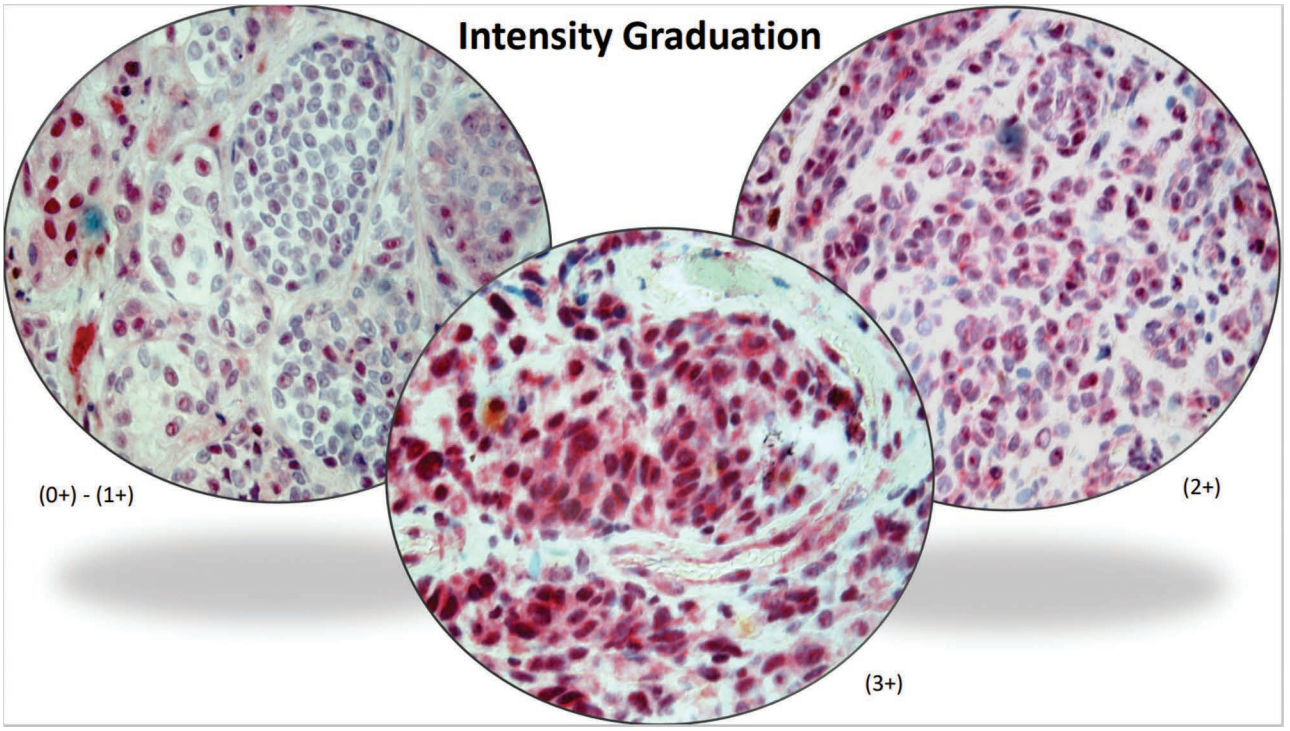
| | | Estimated Differences (95% CI) | *P*-value | |
|---|---|---|---|---|
| Gender | | | 0.75 | |
| Male | ref | | | |
| Female | −0.15 | (−1.08 to 0.78) | | |
| VD supplementation at baseline | | | 0.05 | |
| Yes | −1.27 | (−2.53 to 0.002) | | |
| No | ref | | | |
| Total sun exposure score | | | | Overall *P*-value 0.01 |
| Normal sun exposure | 0.62 | (−0.35 to 1.58) | 0.21 | |
| High sun exposure | 2.4 | (0.77 to 4.04) | 0.004 | |
| Low sun exposure | ref | | | |
| Actinic keratosis | | | 0.11 | |
| Presence | 1.09 | (−0.26 to 2.43) | | |
| No presence | ref | | | |
| Idiopathic guttate hypomelanosis | | | 0.07 | |
| Presence | −1.34 | (−2.77 to 0.09) | | |
| No presence | ref | | | |

Estimated difference, estimated mean difference with the reference category; Ref, reference category.

**Table 3 Correlation of VDR expression with pathological parameters and TNM staging**

| | VDR % nucleus | | VDR % cytoplasm | | VDR H-score | |
|---|---|---|---|---|---|---|
| Mitosis | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| No presence | 21 | 70 (40–90) | 21 | 100 (70–100) | 21 | 100 (85–130) |
| Presence | 251 | 35 (15–70) | 252 | 80 (30–100) | 251 | 95 (30–130) |
| Kruskal–Wallis Test | | $P = 0.002$ | | $P = 0.06$ | | $P = 0.29$ |
| Perineural invasion | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| No presence | 96 | 40 (20–70) | 97 | 80 (35–100) | 96 | 90 (40–120) |
| Presence | 6 | 5 (5–30) | 6 | 20 (10–60) | 6 | 25 (10–60) |
| Kruskal–Wallis Test | | $P = 0.04$ | | $P = 0.06$ | | $P = 0.08$ |
| TNM staging | n | Median (Q1–Q3) | n | Median (Q1–Q3) | n | Median (Q1–Q3) |
| IA | 7 | 80 (60–95) | 7 | 100 (50–100) | 7 | 100 (50–100) |
| IB | 155 | 40 (10–70) | 156 | 80 (30–100) | 155 | 95 (30–120) |
| IIA | 38 | 28 (10–70) | 38 | 65 (20–100) | 38 | 80 (25–125) |
| IIB | 19 | 70 (20–85) | 19 | 50 (30–100) | 19 | 60 (30–170) |
| IIC | 11 | 30 (25–70) | 11 | 60 (40–100) | 11 | 70 (50–140) |
| III | 47 | 40 (20–70) | 47 | 85 (30–100) | 47 | 105 (30–140) |
| Spearman Rho (95% CI) | | −0.01 (−0.13 to 0.11) | | −0.02 (−0.14 to 0.10) | | 0.04 (−0.08 to 0.16) |
| | | $P = 0.84$ | | $P = 0.76$ | | $P = 0.48$ |

**Fig. 1**




Example of staining of CM tissue for calculation of the VDR expression by Histoscore.

brown hair and a fair skin color (Table 1). In multivariable analysis (Table 2) only high total sun exposure remained significant (overall $p_{H\text{-score:}}$ 0.01). Cytoplasmic VDR expression was increased in patients who stated to have a high sun exposure during their life compared to patients with low sun exposure ($p_{H\text{-score}}$: 0.004).

### Correlation VDR expression in the primary tumor with pathological parameters and tumor stage at diagnosis

In univariable analyses, a statistically significant correlation between the absence of mitosis and increased nuclear VDR expression was found ($p_{mitosis}$: 0.002). For perineural invasion, a statistically significant association with decreased nuclear VDR expression was established ($p_{perineuralinvasion}$: 0.04). In addition, a negative correlation between nuclear and cytoplasmic VDR expression and Clark level, Breslow thickness and TNM staging was observed, but these results were statistically NS.

### Association of VDR polymorphisms with VDR expression

Linear regression analysis of 13 *VDR* SNPs with VDR expression demonstrated a significant association between SNP rs2228570 (Fok1) and cytoplasmatic VDR expression. Presence of the A allele was associated with a higher expression of the VDR in the cytoplasm ($P = 0.001$; beta = 11.39, 95% CI [25.73 to −3.26]) and H-score ($P = 0.02$; beta = 16.66, 95% CI [2.74–30.57]). In contrast to the cytoplasmatic expression, no statistically significant associations between the *VDR* SNPs and nuclear VDR expression were found. For details, see Tables 4 and 5.

## Discussion

VDR expression and activation has been of general interest in research in the past. Previous research indicates that the VDR expression can be used as a prognostic marker, since evidence suggests that VDR can have an impact on cancer outcome independently of VD status, possibly mediated by genetics or its subcellular localization [13,14].

Loss of VDR expression in CM was already established by previous groups, both in cell cytoplasm and in the nucleus. Overall results suggest a decrease of VDR expression is associated with progression of melanocytic lesions [15]. Reduction or lack of VDR expression is associated with tumor aggressiveness (defined as advanced tumor stage) and outcome (defined as shorter overall survival and disease-free survival) [8,15]. One plausible explanation for this association, is that expression of VDR is necessary for active VD metabolites to bind and exert anti-carcinogenic effects.

Therefore, this study investigated the influence of clinical/demographic parameters of CM patients and genetic variants of the *VDR* (SNPs) on the VDR

protein expression by localization (cytoplasm, nuclear) in primary CM tissue. Further, we correlated the VDR expression by localization with plural histopathological parameters of primary CM tissue and tumor staging (AJCC 8th edition).

VDR protein expression in primary tumor tissue of this CM population was in general predominant in the cell cytoplasm (mean: 65.4%) compared to the nucleus (mean: 43.7%). This is in line with a previous study [9], where also a predominance of cytoplasmic VDR expression was observed compared to nuclear VDR expression in CM. A cytoplasmic VDR staining pattern was also described in melanoma [16]. The functional impact of cytoplasmic VDR expression is believed to be different from that of nuclear VDR expression. In the classical nuclear pathway, upon binding of 1.25OHD to cytoplasmic VDR and translocation to the nucleus multiple target genes containing VDRE are up-or downregulated. Cytoplasmic VDR expression possibly enables another molecular pathway, through the extracellular-signal regulated kinase pathway (*ERK*). *ERK* is one of the major signaling cascades of the mitogen-activated protein kinase (*MAPK*) signaling pathway. The latter plays a crucial role in melanoma carcinogenesis. Decreased nuclear and high cytoplasmic VDR expressions were associated with malignant progression in terms of dermal invasion and metastasis [16,17]. A failure of VDR nuclear entry caused by *MAPK* activity was proposed as explanation instead of direct inhibition of VDR transcription [16]. CM with high *MAPK* activity together with cytoplasmic VDR expression was associated with worse prognosis [16].

Investigation of the effect of demographic parameters with VDR expression in both cell cytoplasm and nucleus showed a significant correlation between cytoplasmic VDR expression (expressed as H-score) and gender, but not with age. Female patients ($P = 0.04$) had a higher risk to have lower cytoplasmic VDR expression. This finding is in contrast with a previous study [9], where no significant effect of gender on VDR expression was observed. Of the biochemical parameters analyzed, VD supplementation affected VDR expression in our cohort. 25OHD baseline levels and other parameters previously shown to influence VD baseline levels, like season and BMI, did not show a significant correlation [18]. However, these results have to be interpreted with caution since timepoint of VDR expression (time

**Table 4** **Correlation of multiple SNPs of the VDR with cytoplasmic VDR expression expressed in percentages**

| SNP | Reference Allele | N | BETA | SE | L95 | U95 | *P*-value |
|---|---|---|---|---|---|---|---|
| Rs9729 | C | 130 | 1.73 | 4.22 | −6.55 | 10.00 | 0.68 |
| Rs739837 | G | 118 | 0.057 | 4.33 | −8.42 | 8.54 | 0.99 |
| Rs731236 | G | 217 | −2.91 | 3.69 | −10.14 | 4.33 | 0.43 |
| Rs7975232 (Apa1) | C | 92 | 4.30 | 5.27 | −6.03 | 14.64 | 0.41 |
| Rs757343 | T | 218 | 0.94 | 5.15 | −9.15 | 11.04 | 0.85 |
| Rs1544410 (Bsm1) | T | 183 | −3.11 | 4.01 | −10.97 | 4.74 | 0.44 |
| Rs7962898 | C | 130 | 1.98 | 4.22 | −6.30 | 10.26 | 0.64 |
| Rs4760733 | A | 108 | 2.92 | 4.43 | −5.76 | 11.60 | 0.51 |
| Rs7967152 | A | 129 | 1.34 | 4.20 | −6.89 | 9.58 | 0.75 |
| Rs7975128 | A | 128 | 0.61 | 4.63 | −8.46 | 9.69 | 0.89 |
| Rs2228570 (Fok1) | A | 220 | 11.39 | 3.47 | 4.60 | 18.18 | 0.00 |
| Rs10735810 | T | 128 | 6.833 | 4.33 | −1.65 | 15.31 | 0.12 |
| Rs7139166 | G | 129 | 0.45 | 4.64 | −8.63 | 9.536 | 0.92 |

N, number of non-missing genotypes; L95, lower bound of 95% confidence interval; SNP, SNP identifier.
Allele: C, C allele, G, G allele, T, T allele, A, A allele.
BETA: Regression coefficient.

**Table 5** **Correlation of multiple SNPs of the VDR with cytoplasmic VDR expression expressed in H-score**

| SNP | Allele | N | BETA | SE | L95 | U95 | *P*-value |
|---|---|---|---|---|---|---|---|
| Rs9729 | C | 130 | 11.21 | 8.85 | −6.13 | 28.55 | 0.21 |
| Rs739837 | G | 118 | 6.69 | 9.08 | −11.11 | 24.50 | 0.46 |
| Rs731236 (Taq1) | G | 217 | −7.14 | 7.45 | −21.74 | 7.46 | 0.34 |
| Rs7975232 | C | 92 | 1.36 | 9.62 | −17.48 | 20.21 | 0.89 |
| Rs757343 | T | 218 | −5.63 | 10.37 | −25.96 | 14.70 | 0.59 |
| Rs1544410 (Bsm1) | T | 183 | −9.25 | 8.09 | −25.10 | 6.59 | 0.25 |
| Rs7962898 | C | 130 | 10.98 | 8.85 | −6.38 | 28.35 | 0.22 |
| Rs4760733 | A | 108 | 16.77 | 9.22 | −1.31 | 34.85 | 0.07 |
| Rs7967152 | A | 129 | 11.23 | 8.77 | −5.96 | 28.42 | 0.20 |
| Rs7975128 | A | 128 | −4.38 | 9.79 | −23.56 | 14.81 | 0.65 |
| Rs2228570 (Fok1) | A | 220 | 16.66 | 7.10 | 2.73 | 30.57 | 0.02 |
| Rs10735810 | T | 128 | 13.10 | 9.17 | −4.88 | 31.08 | 0.15 |
| Rs7139166 | G | 129 | −11.04 | 9.73 | −30.12 | 8.05 | 0.26 |

L95, lower bound of 95% confidence interval; N, number of non-missing genotypes; U95, upper bound of 95% confidence interval; SNP, SNP identifier.
Allele: C, C allele, G, G allele, T, T allele, A, A allele.
BETA: Regression coefficient.

of CM diagnosis) and timepoint of measured baseline 25OHD levels is not the same, which is a limitation. Reduced cytoplasmic VDR expression (both expressed in percentages and H-score) was observed in patients showing idiopathic guttate hypomelanosis at clinical examination. This parameter was not linked to VDR expression in previous studies. In contrast, high score for intensity of lifetime sun exposure and presence of AK upon physical examination were linked with higher cytoplasmic VDR expression (expressed in percentages and H-score), also new findings. A reduced nuclear VDR expression for CM patients with blond/light brown hair color and fair skin color was found. In our multivariable analysis, only the association of high sun exposure with higher cytoplasmic VDR expression (expressed in H-score) remained statistically significant. The clinical relevance of this finding needs to be further investigated.

Multiple other clinical/demographic parameters were investigated in relation with VDR cytoplasmic and nuclear expression, but we have not found any statistically significant correlation. Previous research [9] looked at various clinical/demographic parameters with VDR expression and also did not observe relevant patient demographics being associated with VDR expression.

*VDR* polymorphisms in the context of CM are mostly studied in terms of CM risk and prognosis [19]. In this study, an association of *VDR* SNPs with VDR expression and localization (cytoplasmic vs. nucleus) was carried out. In this CM population, SNP RS2228570 (Fok1) influenced VDR expression: presence of A allele was associated with elevated cytoplasmic VDR expression. There was no observation of a significant increase in nuclear VDR expression by the Fok1 variant. Fok1 is responsible for a VDR isoform that is longer than the wild-type variant. To our knowledge, this is the first report of an association between Fok1 variant and VDR expression in CM. A previous study also demonstrated an association of Fok1 with increased cytoplasmic VDR expression in breast cancer tissue [20]. This finding supports the idea that genetic variants may influence VDR expression and location of expression. Possible explanations are a prolonged VDR cytoplasmic retention or enhanced cytoplasmic VDR expression via increased transcription by the Fok1 isoform. Fok1 polymorphism is linked to an increased CM risk. However, its precise role in melanoma carcinogenesis and its impact on the biological behavior of the tumor needs to be further explored [21–23].

Previously, a higher VDR expression was linked with CM tumor tissue with low tumor staging and pathology parameters indicative for early progressive tumors [9]. Our univariable analysis for pathological parameters of the primary tumor tissue and VDR expression showed that various histopathological parameters were correlated with VDR expression. An inverse correlation between VDR expression (both nuclear and cytoplasmic) and both Clark level and Breslow thickness were observed. However, these correlations were not statistically significant. In previous studies in 2011 and 2014 [8,14], increased melanoma thickness assessed by Clark level and Breslow thickness showed a decreased nuclear and cytoplasmic VDR expression. From all pathology parameters correlated with VDR cytoplasmic and nuclear expression, we found a significant correlation between the presence of perineural invasion and very low nuclear VDR expression and between the absence of mitosis and relatively high nuclear VDR expression. The presence of perineural invasion is associated with increased risk of local recurrence [24]. Although mitosis is excluded in the 8t AJCC staging as a parameter-indicating stage, it is known as a negative prognostic marker [25]. The correlation of higher mitotic rate with reduced VDR expression was previously documented [5,8].

A correlation of TNM staging, according to the 8$^{th}$ edition of the AJCC classification with nuclear and cytoplasmic VDR expression was carried out. An inverse correlation between T-staging and TNM staging and both VDR expression in the nucleus and cytoplasm was seen, but this finding was not statistically significant. In previous studies conducted [8,26], less advanced CM (TNM staging) showed a higher VDR expression.

The limitation of this study is the timely delay between assessment of VDR expression (timepoint of CM diagnosis) and the timepoint of measured baseline 25OHD levels (time of inclusion). This limitation makes the interpretation of the associations of this parameter with VDR expression difficult (and might explain the lack of statistically significant correlations of some parameters with VDR expression). In addition, we investigated several factors and no correction for multiple testing was applied. Hence, results must be interpreted with caution.

Strengths of our study are the VDR protein expression analysis, which was separately done for cytoplasmatic and nuclear localization, the detailed phenotyping of our study population, and the correlation of several VDR polymorphisms with VDR expression in a large prospective recruited genetically uniform melanoma population (97% of European ancestry).

Based on our findings, one can assume, a different biological function of nuclear and cytoplasmic VDR expression. While increased nuclear VDR expression and its associated pathway is clearly a positive prognostic parameter, the biological function and mechanism of increased VDR protein expression in the cytoplasm is less clear. A possible hypothesis for an elevated cytoplasmic VDR expression is an interrupted transport of VDR from the cytoplasm to the nucleus through interaction with the MAPK pathway or the direct influence of certain SNPs on VDR transcription. A mechanistic link between MAPK activity and cytoplasmatic VDR expression could potentially unravel

VDR expression as a predictive marker for response to MAPK pathway inhibitory treatments.

The detailed phenotyping of this study population, based on validated questionnaires and physical examination, demonstrates for the first time that clinical parameters indicative of sun damage and sensitivity to sun damage affect VDR expression in primary CM tissue.

*VDR* polymorphisms are associated with VDR expression in primary CM tissue with an association of Fok1 with increased cytoplasmatic expression. Further research is needed to study the relevance of this finding and the underlying mechanism (with respect to CM outcome, which will be part of our future study aims in this melanoma population).

This study together with previous findings from other groups, show that VDR expression is associated with prognostic histological parameters in CM and more research is needed to explore the biology and the full prognostic/predictive impact of VDR as putative biomarker.

### Conclusion

VDR expression, in both cell nucleus and cytoplasm of CM tissue, can be influenced by both clinical/demographic and genetic variants. VDR expression may have a potential value as a prognostic marker.

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### Conflicts of interest

There are no conflicts of interest.

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